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Physiological and Antioxidant Responses of Germinating Mung Bean Seedlings to Phthalate Esters in Soil*[1]

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ABSTRACT

Single phytotoxicity of two representative phthalate esters (PAEs), di-*n*-butyl phthalate (DnBP) and bis(2-ethylhexyl) phthalate (DEHP), was tested in mung bean (*Vigna radiata*) seedlings germinated for 72 h in soils spiked with varying concentrations (0–500 mg kg^{-1} soil) of DnBP or DEHP. PAEs added at up to 500 mg kg^{-1} soil exerted no significant effect on germination but both pollutants significantly inhibited root elongation ($P < 0.01$), DEHP inhibited shoot elongation ($P < 0.01$) and DnBP depressed biomass on a fresh weight basis ($P < 0.05$). Seedling shoot and root malondialdehyde (MDA) contents tended to be stimulated by DnBP but inhibited by DEHP. However, increases in superoxide dismutase, peroxidase, ascorbate peroxidase and polyphenol oxidase activities, as well as glutathione (GSH) content, were induced at higher concentrations (*e.g.*, 20 mg kg^{-1}) of both compounds. Accumulation of proline in both roots and shoots and the storage compounds, such as free amino acids and total soluble sugars, in whole plant was induced under the stress exerted by both PAEs. The general responses of mung bean seedlings indicated higher toxicity of DnBP than DEHP on primary growth, during which root elongation was a more responsive index. MDA and GSH were more sensitive parameters in the roots than in the shoots and they might be recommended as physiologically sensitive parameters to assess the toxicity of PAE compounds in soils in future long-term studies.

Key Words: glutathione, malondialdehyde, phytotoxicity, root elongation, storage compounds

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INTRODUCTION

Phthalate esters (PAEs) are a group of diesters primarily used as plasticizers and are readily released to different environmental media as contaminants because of their weak binding forces with polyolefin molecules (Staples *et al.*, 1997). Reported as endocrine disrupting compounds (EDCs), six PAEs have been nominated by USEPA as priority pollutants. The PAEs compounds di-*n*-butyl phthalate (DnBP) and bis(2-ethylhexyl) phthalate (DEHP) have also been classified by the European Union (EU) as priority pollutants. DnBP and DEHP are the dominant PAEs in the contaminated soil at an electronic waste dismantling area in East China and in the urban soils of Guangzhou in South China (Liu *et al.*, 2009; Zeng *et al.*, 2009), and their concentrations are sometimes 2–3 orders of magnitude higher than those reported in agricultural soils at Roskilde in Denmark (Vikelsøe *et al.*, 2002). DnBP and DEHP have raised wide public concerns because of their potential carcinogenicity and mutagenicity in humans after long-term contact at low concentrations (Hu *et al.*, 2007).

Toxic effects of DnBP and DEHP in contaminated soils are of concern in addition to their toxicity to animals. Their effects on urease, phosphatase, catalase, microorganisms, fauna and the microbial community in soil were significantly greater compared with those of the pristine controls (Chen *et al.*, 2004; Gao and Chen, 2008; Xie *et al.*, 2009; Gao, 2010). DnBP and DEHP in soil may also affect the quality of vegetables such as peppers (*Capsicum* spp.) by decreasing the content of vitamin C in the fruit (An *et al.*, 1999; Yin *et al.*, 2002). Phytotoxicity tests using higher plants are frequently employed for toxicity evaluation of heavy metals (Chen *et al.*, 2010), but there are few for orga-

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nic pollutants such as PAE compounds. Toxic effects of DnBP on the growth of Chinese cabbage (*Brassica rapa* var. *chinensis*) have been reported, including significant accumulation and differences in expression of six protein spots in leaf tissue under high concentrations of the pollutant (Liao *et al.*, 2009).

The germination stage is crucial for future vegetative and reproductive growth of different higher plant species under the stress of different pollutants. Although germination and root elongation tests have been criticized occasionally as insensitive and representing only a single life stage, they are also supported because the roots are in direct contact with the contaminants in soil during the initial growth stages (Kapustka, 1997). Biomass has been found to be a better indicator for pollutant toxicity than root or shoot elongation (Shi and Cai, 2009). Antioxidant enzyme status is important for understanding EDC effects, including superoxide dismutase (SOD) and peroxidase (POD) in mediating responses to stressors (Zhou *et al.*, 2010; Boojar and Tavakkoli, 2011). Proline, free amino acids (FAA) and total soluble sugars (TSS) can be induced under adverse environmental stresses including drought, salinity and low temperatures (Xu *et al.*, 2001; Liu *et al.*, 2007; Kocsy *et al.*, 2011). Toxicity to individual plants as revealed by the germination tests and effects on antioxidant enzymes and other critical compounds provide useful evidence in assessing the toxicity of organic pollutants to sensitive terrestrial dicotyledonous plant species.

The present study examined the phytotoxicity of the two typical PAE pollutants DnBP and DEHP in soil to mung bean (*Vigna radiata*). Their effects on initial plant growth (germination, root elongation, shoot elongation and biomass) and changes in the activity or content of different enzymes, critical amino acids and some storage compounds such as FAA and TSS were examined. The aim was to compare the potential toxic effects of typical PAE pollutants in soil and to make preparations for further prediction of toxic threshold concentrations. More importantly, it was hoped that the study would provide fundamental data for further health risk assessment and ecosystem effects in China, where soil pollution by PAE compounds is increasing due to the use of plastic films in agriculture.

MATERIALS AND METHODS

Chemicals

DnBP (99.1%) and DEHP (99.6%) were obtained from AccuStandard, Inc., New Haven, USA. The nitro blue tetrazolium (NBT), *L*-methionine (L-Met), ascorbic acid (AsA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), proline, alanine, sulfosalicylic acid, ninhydrin, catechol, anthrone, polyvinylpyrrolidone (PVP), monosodium orthophosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), hydrogen peroxide (H_2O_2), riboflavin, ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2), sodium hydrate (NaOH), glacial acetic acid and acetone used were all analytical reagents purchased from the National Pharmaceutical Group Chemical Reagent Co., Ltd., Shanghai, China. The glutathione (GSH) assay kit (catalog number A006) was purchased from the Jiancheng Bioengineering Institute, Nanjing, China.

Soil and plant seed preparation

The test soil, collected from a comparatively uncontaminated area at Qixia in Nanjing, Jiangsu Province, East China, is an Alfisol according to the USDA soil classification. The soil had a pH (in water) of 7.4, a clay content of 1.67 g kg^{-1}, an organic matter content of 14.6 g kg^{-1}, and available nitrogen, phosphorus and potassium concentrations of 96.8, 14.4 and 102.8 mg kg^{-1}, respectively. The soil was passed through a 2-mm sieve before use and the background concentrations of the two target pollutants DnBP and DEHP were determined to be 142.6 ± 4.2 and 194.5 ± 1.1 μg kg^{-1} soil, respectively.

Batches of soil samples were adjusted to concentrations of DnBP or DEHP of 0, 5, 20, 100 and 500 mg kg^{-1} soil by spraying aliquots of soil with stock solutions of PAEs in acetone. After the acetone had evaporated off, each spiked soil sample was mixed thoroughly before use.

According to the recommendations of the Organization for Economic Co-operation and Development (OECD, 1984) for test species in terrestrial environmental assessment and to promising results from previous studies (Yusuf *et al.*, 2011), seeds of mung bean (*Vigna radiata*) obtained from the Chinese Academy of Agricultural Sciences were selected as our test species for terrestrial environmental assessment. The seeds were surface sterilized by immersion in 10% (v/v) sodium hypochlorite solution for 10 min (USEPA, 1996), rinsed three times with deionized water and soaked in deionized water for 2 h before use. Plastic equipment was avoided throughout the procedure to eliminate background PAE contamination. All the glass Petri dishes were washed and baked in an oven at 400 °C before use.

Inhibition experiment

Samples (250 g) of test soil spiked with different concentrations of DnBP or DEHP from 0 (control) to 500 mg kg^{-1} soil were placed in 150 mm × 20 mm glass Petri dishes with the soil at about 70% water holding capacity. Each treatment was set up in quadruplicate and 50 pre-treated swollen seeds of uniform size of the test species were sown with equal spacing in each Petri dish. The dishes were closed, sealed with tape and placed in the dark in a growth chamber at 25 ± 1 °C for 72 h before germination was halted. The germination status of each treatment was checked, seedling root and shoot lengths were measured with a millimeter ruler, and the biomass (fresh weight, FW) in each dish was determined by weighing. Root length was defined as the length from root tip to root radicle. Seed germination was defined as a root length of 5 mm or more (Wang *et al.*, 2001).

Determination of some biochemical indices

Analyses of all indices were performed in triplicate on both shoots and roots of seedlings except FAA and TSS, both of which were determined on whole plants. Induced oxidative damage was estimated by measuring malondialdehyde (MDA) levels. About 0.5 g of fresh sample was homogenized in 5 mL of 0.1% (w/v) TCA solution. After centrifugation (15 min, 6 000 r min^{-1}), 2 mL of the supernatant was added to 2 mL of 0.5% (w/v) TBA in 20% (w/v) TCA solution and heated in a boiling water bath for 30 min. After fast cooling on ice, the mixture was centrifuged at 6 000 r min^{-1} for 5 min. The absorbance of the supernatant at 532 and 600 nm was read to calculate the MDA content (Amor *et al.*, 2005).

For SOD assay, fresh plant samples of about 0.5 g were ground in a mortar to extract with ice-cold 50 mmol L^{-1} potassium phosphate buffer (PBS) (pH 7.8). The homogenates were centrifuged at 6 000 r min^{-1} at 4 °C for 20 min and the supernatant was collected. SOD activity was determined following the method of Giannopolitis and Ries (1977) by measuring its ability to inhibit the photochemical reduction of NBT. One unit of SOD activity is defined as the amount of enzyme inhibiting 50% of the initial reduction of NBT under light. The enzymatic activity was expressed as units (U) mg^{-1} FW.

For POD assay, enzyme samples were prepared in the same way as for SOD but using PBS at pH 7.0. POD activity was determined by the method of Noreen and Ashraf (2009) with some modification, based on the observation that POD can catalyze the transformation of guaiacol to tetraguaiacol (a brown colored product) in the presence of H_2O_2. Absorbance changes in the reaction solution at 470 nm were determined after every 60 s. POD activity was expressed by change in absorbance as U g^{-1} FW min^{-1}.

Using the same enzyme extraction method as for SOD, ascorbate peroxidase (APX) assay was performed using the method of Koricheva *et al.* (1997) with 3 mL reaction mixture consisting of 50 mmol L^{-1} PBS (pH 7.0), 0.3 mmol L^{-1} AsA, 0.06 mmol L^{-1} H_2O_2, 0.1 mmol L^{-1} EDTA-Na_2 and 0.1 mL enzyme sample. The reaction was initiated by the addition of H_2O_2 and the decrease in absorbance at 290 nm within 30 s was measured with a UV spectrophotometer.

The procedure for the GSH assay followed the instructions of the GSH assay kit and was based on the production of a yellow compound. This yellow compound with an absorbance at 405 nm was formed following the reaction between GSH and dithio-bis-nitrobenzoic acid. An enzyme solution was prepared with about 0.2 g sample in 0.1 mol L^{-1} PBS (pH 7.5) containing 0.5 mmol L^{-1} EDTA-Na_2. Then 0.1 mL of the enzyme solution was mixed with 0.1 mL test buffer and 0.025 mL color-developing buffer in 96-well microtiter plates. The optical density was read at 405 nm 5 min after mixing. GSH content was calculated using a standard curve.

Polyphenol oxidase (PPO) is an enzyme that catalyzes the O_2-dependent oxidation of monophenols or *o*-diphenols. Fresh plant samples were ground in a mortar with ice-cold 0.1 mol L^{-1} PBS (pH 7.8) containing 1% (w/v) solid PVP. After centrifugation at 15 000 × *g* for 15 min at 4 °C, the supernatant was used for the PPO assay. The reaction was initiated by the addition of 30 μmol L^{-1} catechol. PPO activity was measured by the disappearance of catechol spectrophotometrically at 525 nm every 30 s for 5 min at 25 °C.

Proline content was determined by the method of Li *et al.* (1995) with modification. A fresh sample of about 0.5 g was homogenized with 5 mL of 3% (w/v) sulfosalicylic acid and incubated at 100 °C for 10 min. Then, 2 mL of the supernatant was added to a mixture of 2 mL glacial acetic and 2 mL 2.5% (w/v) acidic ninhydrin before boiling at 100 °C for a further 25 min. After the solution cooled, 5 mL of toluene was added for extraction, prior to reading the absorbance of the upper red layer (toluene) at 520 nm and calculating the content of proline (mg g^{-1} FW).

The total protein concentration in soluble enzyme extracts was determined using the Bradford (1976) assay. FAA content was estimated following the method of Shukla *et al.* (2002) with minor modification. Fifty

mL ice-water bathed ground fresh samples were stirred in 5 mL 10% (v/v) acetic acid overnight and distilled water was added to adjust the total volume to 50 mL. After filtration, 2 mL extract, 3 mL 1% (w/v) ninhydrin (in 0.5 mol L^{-1} citrate buffer, pH 5.5) and 0.1 mL 1% (w/v) ascorbic acid were added and mixed before heating in a boiling water bath for 15 min. After cooling, the FAA content was then determined by the absorbance at 570 nm. Alanine was used as the standard for quantification of the FAA content. TSS content was estimated according to Moya *et al.* (1993) with slight modification. Eighty mL 80% (w/v) ethanol was added to 1.0 g of fresh sample for boiling water bath extraction at 80 °C for 30 min. The extraction was repeated twice and the mixture centrifuged at 12 000 × *g* for 10 min. All the supernatants were combined and made up to a final volume of 25 mL with 80% (w/v) ethanol. Ten mL extract was steamed to dryness over a boiling water bath. After addition of 0.5 mL of H_2SO_4-anthrone reagent, the TSS content was determined by spectrophotometry at 620 nm. Glucose was used as the standard for quantification of the TSS content.

Statistical analysis

Statistical analyses were conducted using SPSS 15.0 for windows. All results are presented as mean values ± standard errors of the means of three replicates from three Petri dishes. Relationships among inhibition of germination rate, shoot elongation, root elongation and biomass of mung bean and PAE concentrations were analyzed by regression analysis in order to assess the effects of PAEs on the test plant seedlings. Differences were assessed by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) test, and the probability level of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Seed germination, shoot elongation, root elongation, and biomass

Results of the regression analysis showed that there was little effect of either pollutant on the germination rate of mung bean as the target pollutants increased to the highest concentration studied (500 mg kg^{-1} soil), with the R values under the two PAE compounds being 0.2237 and 0.3533, far less than 0.5 (Table I). In contrast, highly significant inhibition of root elongation ($P < 0.01$) under exposure to both DnBP and DEHP, highly significant retardation of shoot elongation ($P < 0.01$) under DEHP and significant depression of biomass ($P < 0.05$) by DnBP were observed. Root elongation is considered the most sensitive parameter in the evaluation of phytotoxicity of both DnBP and DEHP, with the lowest 50% effective concentrations (EC_{50}) of 4 016 and 3 969 mg kg^{-1} soil, respectively, which are the values not within the range used in this study. However, biomass was the most sensitive parameter for the evaluation of DnBP toxicity, with an EC_{50} value of 1 559 mg kg^{-1} soil. In contrast, shoot elongation was the most sensitive indicator of DEHP toxicity (EC_{50} = 16 550 mg kg^{-1} soil).

The lack of an effect on germination rate may be explained by the fact that mung bean is a dicotyledonous species which can derive most of its nutrition from its hypertrophic cotyledons rather than from the soil during the initial phases of plant growth. Consequently, no significant differences were found in germi-

TABLE I

Parameters[a)] of regression analysis on the relationships of the inhibition of germination rate, shoot elongation, root elongation and biomass of mung bean seedlings with the concentration of single di-*n*-butyl phthalate (DnBP) or bis(2-ethylhexyl) phthalate (DEHP) in the test soil

Compound	End point[b)]	*a*	*b*	*R*	EC_{50}[c)]
					mg kg^{-1} soil
DnBP	Germination rate	−0.0031	96.33	0.2237	31 235
	Shoot elongation	0.0000	1.13	0.3899	∞
	Root elongation	−0.0010	3.52	0.9513**	4 016
	Biomass	−0.0080	11.97	0.8826*	1 559
DEHP	Germination rate	−0.0023	97.88	0.3533	42 774
	Shoot elongation	−0.0010	11.55	0.9581**	16 550
	Root elongation	−0.0010	3.47	0.9607**	3 969
	Biomass	0.0000	1.10	0.5814	∞

*, **Significant at the 0.05 and 0.01 levels of probability, respectively.
[a)] *a* and *b* are the coefficients of the regression equation, and *R* is the correlation coefficient.
[b)] The data are the average values of 200 seeds.
[c)] 50% effective concentration.

nation percentage even when the seeds were exposed to a range of concentrations of the two PAEs. Generally, with few exceptions, root elongation was the most sensitive end point (Hillis *et al.*, 2011). The EC_{50} value of biomass under DnBP was the lowest in all the evaluated growth parameters. The severe stunting in biomass during the initial growth stages may represent critical damage for subsequent plant development and growth. In addition to the highly significant inhibition of root elongation, DnBP showed higher phytotoxicity during this stage of mung bean development taking into consideration of all the growth parameters. Furthermore, root elongation might be better than shoot elongation as a sensitive index for evaluating the phytotoxicity of PAE compounds.

Antioxidases

Antioxidant status, an indicator of physiological equilibrium, is critically important for the understanding of the responses of organisms to contaminated environments. The increases in MDA contents were induced with exposure to DnBP but not to DEHP (Fig. 1). The activities of SOD, POD, APX, GSH and PPO all increased with exposure to both pollutants.

In their review, Bernanke and Köhler (2009) stated that EDCs cause increases in oxygen free radical levels and disturb the antioxidative balance in wildlife vertebrates, with similar effects on plants. Antioxidant enzymes and certain metabolites inducible in oxidative stress play a vital role in the adaption and ultimate survival of plants under stress conditions. Generally, the formation of MDA in plants is considered to reflect oxidative stress as a result of lipid peroxidation. Usually the content of MDA under oxidative stress will increase as one of the protective mechanisms, and the consumption of MDA indicates less severe damage to plant organs. The coincidence of increasing MDA caused by DnBP exposure and decreasing MDA due to DEHP suggests that DnBP is more toxic than DEHP to mung bean. The more severe effects on MDA in the roots may also indicate the sensitivity of the roots and the value of MDA content as an indicator of toxicity. Both POD and SOD are extremely important antioxidant enzymes in the understanding of stress response me-

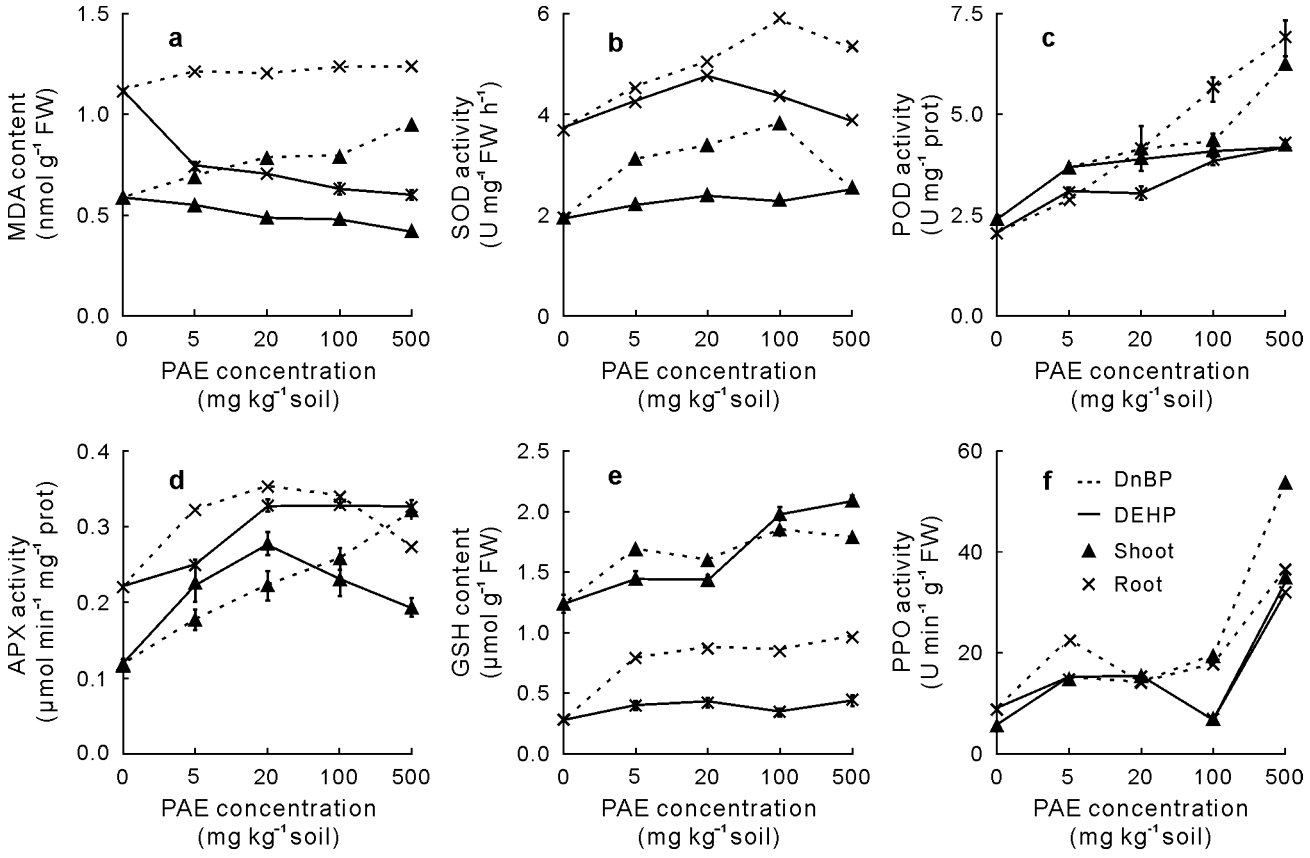

Fig. 1 Malondialdehyde (MDA) content (a), superoxide dismutase (SOD) activity (b), peroxidase (POD) activity (c), ascorbate peroxidase (APX) activity (d), glutathione (GSH) content (e) and polyphenol oxidase (PPO) activity (f) of shoots and roots of mung bean seedlings exposed to 0 (control)–500 mg kg^{-1} soil of two phthalate ester (PAE) compounds, di-*n*-butyl phthalate (DnBP) and bis(2-ethylhexyl) phthalate (DEHP), after germination for 72 h. Values are means of three replicates and bars represent standard errors of the means. FW = fresh weight. U = units.

chanisms in cells in connection with the production of reactive oxygen species (ROS). The elevation of POD activity reflects an accumulation of ROS level which can also increase the lipid peroxidation measured by MDA content (Chiou *et al.*, 2003). Although the MDA content in mung bean exposed to DEHP did not yet exhibit any discernible increase (Fig. 1a), it is quite possible that a subsequent increase would have occurred after a longer period of vegetative growth as nutrients were taken up from the soil. SOD activity in mung bean tended to increase with increasing concentrations of both pollutants (Fig. 1b), suggesting the timely response of mung bean to adverse conditions. DnBP caused a greater change in SOD activity than DEHP, suggesting greater toxicity of DnBP. POD activity showed some similarity with SOD activity, with a much more readily observed increase under DnBP stress in the roots (Fig. 1c). In this respect, POD activity may be considered to be a more sensitive parameter than SOD. Antioxidation and detoxification comprise the first line of defense to environmental stress (Wilhelm Filho, 2007), but increasing pollutant concentrations may lead to the breakdown of these processes and the stimulation of other responses (Zhou *et al.*, 2010). Reduced levels of SOD activity indicate a loss of protective capacity against cellular superoxide toxicity which may be caused by consumption of SOD over the short term and result in a transient shortage of SOD.

The APX activity responses were similar to those of SOD. The original activity in the roots was higher than that in the shoots and the initial stimulation of APX activity under both pollutants was obvious at 5 mg kg^{-1}. Shoot APX activity was higher at lower concentrations (less than 20 mg kg^{-1} soil) of DEHP but reached the highest level at 500 mg kg^{-1} soil of DnBP (Fig. 1d). This increase may be explained by the minimization of excessive levels of H_2O_2 in the plants because APX is one of the two major scavengers of H_2O_2. APX is present throughout the cell and has high substrate affinity when ascorbate acts as a reductant. APX showed higher activity in the roots, which does not agree with the observation that the APX activity can increase more in leaves than in roots in response to heavy metal stress in other plant species (Zhang *et al.*, 2008, 2010) but is similar to our former conclusion that roots were more sensitive than shoots. The results also have some credence because during germination APX plays an important role in avoidance of H_2O_2 toxicity and the roots may possess a higher capacity to scavenge excess H_2O_2 during contact with contaminated soil.

Glutathione was induced in the shoots and roots under different treatments but the primary content was higher in the shoots (Fig. 1e). The appearance of sensitivity in the roots accords with our earlier conclusions on SOD, POD and APX because significant stimulation of GSH was observed at 5 mg kg^{-1} soil of DnBP or DnBP for the roots and about 100 mg kg^{-1} soil for the shoots. GSH is an important component that is involved in detoxification in the defense systems of a range of plant species (Hu *et al.*, 2009). Increasing GSH content indicates that both pollutants may become harmful to the test plants as their concentrations increase. Nevertheless, the more pronounced response of mung bean to DnBP stress after exposure for 72 h might be interpreted as a higher sensitivity of the plant species to DnBP and a higher toxicity of DnBP. However, observations over longer periods are required to fully elucidate the responses of plants to PAE contamination.

Polyphenol oxidase is a general name for tyrosinase, catechol oxidase and laccase, of which only catechol oxidase tends to exist in plants. Although it has been over a hundred years since the first report of PPOs in plants (Bertrand, 1896), a complete knowledge of their role in plants has not been resolved until now. However, important functions of plant protection against pathogens (Constabel and Ryan, 1998) have been well recognized and their activities may increase in response to both biotic and abiotic stresses (Kwak *et al.*, 1996). It has been stated that PPOs are defense-related proteins whose activities are most intensively induced in tomato plants submitted to either wounding and/or to methyl jasmonate vapour treatment (Constabel *et al.*, 1995). In the present study, the increasing trend of PPO activities in all treatments might indicate phytotoxicity of both DnBP and DEHP. In addition, exposure to DnBP induced more PPO activity than DEHP, especially in the shoots, perhaps due to the presence of PPO in both chloroplasts and mitochondria. However, PPO activity in roots exposed to 500 mg kg^{-1} soil of DnBP exceeded that in shoots and represented the highest PPO activity observed in the experiment (Fig. 1f). In general, when DnBP and DEHP phytotoxicities to mung bean were compared, MDA and GSH in the roots appeared to be the most sensitive parameters.

Critical proteins

Proline content was higher in shoots than roots in the control (Fig. 2a). The roots were more susceptible to PAEs because they showed significant reactions at 5 mg kg^{-1} DnBP or DEHP compared with the shoots (Fig. 2a).

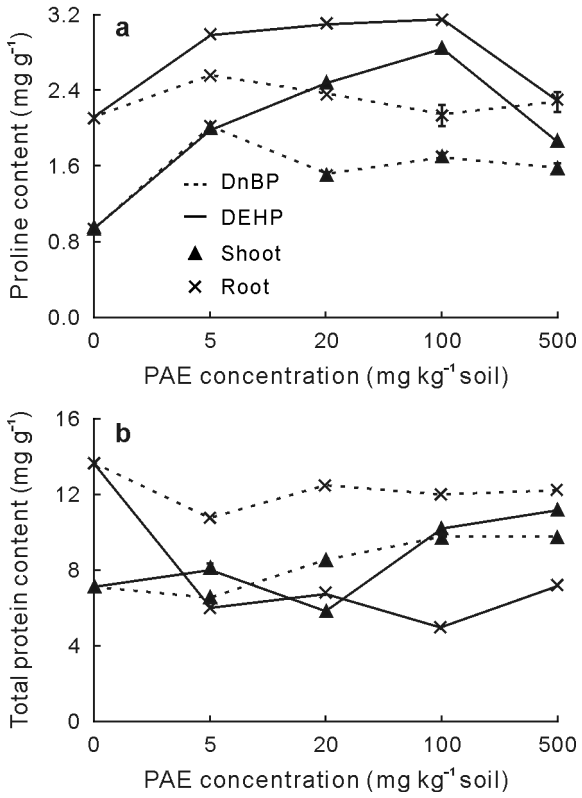

Fig. 2 Proline content (a) and total protein content (b) in shoots and roots of mung bean seedlings exposed to 0 (control)–500 mg kg^{-1} soil of two phthalate ester (PAE) compounds, di-*n*-butyl phthalate (DnBP) or bis(2-ethylhexyl) phthalate (DEHP), after germination for 72 h. Values are means of three replicates and bars represent standard errors of the means.

Biomarkers represent changes from the molecular to the organism level that can be related to toxic effects of and/or exposure to chemical contaminants. In addition, biomarker responses occur prior to alterations at the population and community levels so that they can be predictive and anticipatory. The proline content of plants was originally investigated as a response to climatic change (Treichel *et al.*, 1984) and heavy metal contamination has also been reported to be able to induce the formation of proline in plants (Rai *et al.*, 2004; Sharma and Dietz, 2006). Effects of pesticide stress on free proline content in *Euonymus japonica* have been investigated and the results suggest that the free proline content increased substantially 1 to 10 d after pesticide application (Qu *et al.*, 2006). The proline contents in both roots and shoots of mung bean were promoted across all pollutant treatments (Fig. 2a), which is in accordance with earlier studies (Qu *et al.*, 2006). In normal conditions the content of free proline in plants is low. However, under stress conditions such as drought, low temperatures and high salinity, the content of free proline can increase substantially. The accumulation of proline can be ascribed to an enhancement of proline synthesis, an inhibition of proline oxidation and a decline in total protein synthesis accompanying the inhibition of proline synthesis (Wang, 2000). Proline can protect plant cells from adverse conditions by maintaining the integrity of cell membranes and preventing water loss from the cells (Zhang and Li, 2003). The content of proline in plant cells and tissues under contaminated conditions is therefore an important topic of investigation.

Storage compounds

The contents of total protein in roots and shoots and FAA and TSS in whole plant of mung bean all tended to be stimulated with exposure to both target pollutants, especially FAA and TSS under DnBP treatment (Figs. 2b, 3). Starratt and Lazarovits (1999) reported that low levels of the herbicide trifluralin induced elevated levels of free amino acids in melon seedlings. It has also been found that TSS can be induced under stress of drought, salinity and low temperatures together with increases in both FAA and proline (Ren *et al.*, 2001; Xu *et al.*, 2001; Liu *et al.*, 2007). With increasing stress from organic pollutants such as

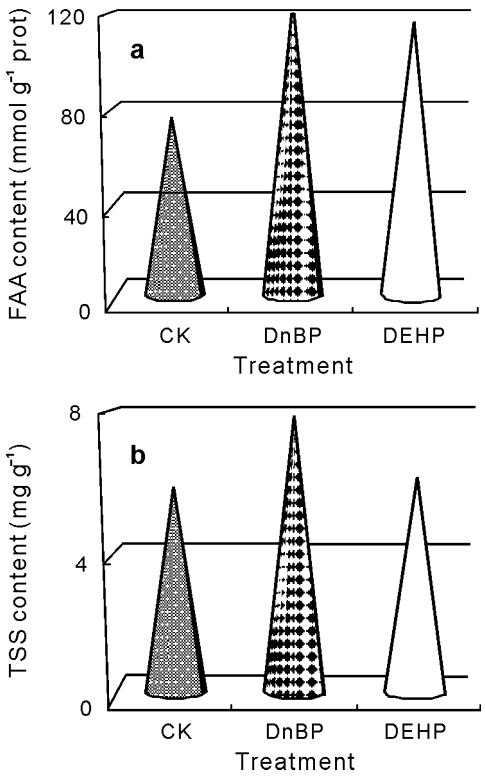

Fig. 3 Mean total soluble sugar (TSS) and free amino acid (FAA) contents in whole plant of mung bean seedlings exposed to 0 (control, CK) or 500 mg kg^{-1} soil of di-*n*-butyl phthalate (DnBP) or bis(2-ethylhexyl) phthalate (DEHP), after germination for 72 h.

PAEs, the plant may respond by increasing its storage compounds during germination. An increase in storage compounds is the response of the self-defense mechanism of a plant and may represent a rapid response before the plant can synthesis adequate protective enzymes.

Other storage compounds such as lipids were not included in the present study but they deserve to be included in the analysis of phytotoxicity from organic pollutants. The internal relationships between seed lipid content, accumulation of PAEs in seedlings and the phytotoxicity of PAEs at the seedling stage are still not fully understood and need further investigation.

CONCLUSIONS

Although no visible difference in seed germination rate was observed in mung bean exposed to DnBP or DEHP, root elongation inhibition was noted as a sensitive parameter of phytotoxicity. Antioxidant enzyme responses indicated that DnBP was more phytotoxic than DEHP. Proline, FAA and TSS contents increased in response to adverse conditions, thus confirming their value in phytotoxicity experiments. MDA and GSH were more sensitive parameters in the roots than in the shoots and they could be selected as physiologically sensitive parameters for the assessment of PAE toxicity in soil. Mung bean can be recommended for further investigation into the phytotoxicity of PAE compounds due to its high sensitivity. Overall, the physiological indices over the whole growth period of the plants merit future investigation to reveal chronic phytotoxicity and detailed trauma due to DnBP and DEHP over the longer term.

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